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(54) Title: INHIBITING PROLIFERATION OF ARTERIAL SMOOTH MUSCLE CELLS

(57) Abstract

The present invention provides methods and compositions for inhibiting the proliferation of smooth muscle cells at a site of vascular injury. The methods include intravascular administration of a reactive compound to the site of injury, without the requirement for activation or sustained release of the compound.

INHIBITING PROLIFERATION OF ARTERIAL SMOOTH MUSCLE CELLS

TECHNICAL FIELD

This invention relates to methods, compounds and devices for inhibiting smooth muscle proliferation at a site of injury.

BACKGROUND

Arteriosclerosis is a class of diseases characterized by the thickening and hardening of the arterial walls of blood vessels. Although all blood vessels are susceptible to this serious degenerative condition, the aorta and the coronary arteries serving the heart are most often affected. Arteriosclerosis is of profound clinical importance since it can increase the risk of heart attacks, myocardial infarctions, strokes, and aneurysms.

The traditional treatment for arteriosclerotic vessels has been coronary bypass surgery. More recently, however, vascular recanalization procedures for treating arteriosclerotic vessels have been developed. These procedures involve using intravascular devices threaded through blood vessels to the obstructed site, including for example, percutaneous transluminal angioplasty (PTA), also known as balloon angioplasty. Balloon angioplasty uses a catheter with a balloon tightly packed onto its tip. When the catheter reaches the obstruction, the balloon is inflated, and the atherosclerotic plaques are compressed against the vessel wall. A shortcoming of this and other intravascular procedures, however, is that in a number of individuals some of the treated vessels restenose (*i.e.* the vessels narrow) by six months post-angioplastic treatment. The restenosis is thought to be due in part to mechanical injury to the walls of the blood vessels caused by the intravascular device.

The walls of most blood vessels are composed of three distinct layers, or tunics, surrounding a central tubular opening, the vessel lumen. The innermost layer that lines the vessel lumen is called the tunica intima. The middle layer, the

and then activated by a visible light source. U.S. Patent No. 5,354,774. Another approach is the use of radiation-emitting catheters or guide wires, which can cause damage to nucleic acid and inhibit smooth muscle cell proliferation. Each of these methods, however, requires an added level of complexity, namely incorporation of the agent on or within a sustained release formulation, photoactivation using a complex intravascular light source, or delivery of a radiation dose which requires the presence of a radiologist and presents exposure hazards to the attending personnel.

A need therefore exists for safer and less complex methods for inhibiting smooth muscle cell proliferation at a site of injury following vascular recanalization procedures.

SUMMARY OF THE INVENTION

The present invention includes methods for inhibiting smooth muscle cell proliferation in a blood vessel subjected to recanalization. The methods include administration of an alkylating compound, not in a sustained or controlled release process, to an individual undergoing vascular recanalization, by local delivery to the vascular recanalization site.

Advantages of the present invention include the following. First, because the compounds for use in the methods of the invention are rapidly taken up by cells, and they react rapidly and permanently with cellular nucleic acid, these compounds need not remain long at the site of administration to be effective. Unlike sustained exposure processes, the compounds and methods of the present invention have a sustained effect with a very short exposure time. Thus, the problem of insufficient residence time associated with local delivery of previously reported agents for inhibiting smooth muscle cell proliferation is overcome. In addition, because the compounds for use in the methods of the invention are rapidly taken up by cells, and they react rapidly with cellular nucleic acids, their toxicity can be significantly reduced by shorter exposure times and lower concentrations.

group, capable of reacting to form a covalent bond with a nucleic acid. Preferred effector groups include mustard, mustard intermediates and mustard equivalents. A particularly preferred class of mustards are aliphatic mustards.

The alkylating compounds for use in the methods of the invention may also be provided in a form that includes an effector group covalently bonded to a nucleic acid binding ligand, also referred to as an anchor group. Anchor groups may include, for example, intercalators, such as acridines and acridine derivatives. A preferred compound for use in the methods of the invention is quinacrine mustard.

In addition, the alkylating compounds for use in the methods of the invention may be provided in a form that includes an anchor group covalently bonded to a frangible linker, which is covalently bonded to the effector group. A preferred compound for use in the methods of the invention in this class is β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester.

The methods of the invention include administering the compounds for use in the invention by local delivery. In one embodiment, the compounds for use in the methods of the invention may be locally delivered by a catheter system. In a preferred embodiment, the compounds for use in the methods of the invention may be locally delivered by an infusion sleeve catheter. The compounds for use in the methods of the invention may be administered before, during or after the recanalization procedure.

In addition, the method may also involve the administration of a quencher. In one embodiment the quencher is a thiol type quencher, including for example, glutathione or thiol sulfate. Although there are naturally occurring quenchers that already exist in certain biological fluids, glutathione in the blood for example, it may be desirable in certain circumstances to increase their concentration or to administer other quenching agents. The quencher may be added by local or systemic delivery. If the quencher is administered systemically, the quencher may be added prior to, in proximal time with, or after the administration of the alkylating compounds for use in the methods of the invention. If the quencher is

(EDGF). These chemicals, and in particular PDGF, apparently play a role in inducing smooth muscle cell proliferation which in turn produces substantial quantities of intercellular substances which build up, and the intima begins to thicken. Methods for measuring the proliferating ability of smooth muscle cells are known to those of skill in the art, including for example, assays that measure the uptake of [^{3}H]-thymidine by smooth muscle cells, as described in March et al., *Circulation*, 87:184-191 (1993), and immunoassays for detecting human cytokines, including for example, interleukin 1β (IL-1β), interleukin 8, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and endothelin-1.

The methods of the present invention are directed at inhibiting restenosis caused, for example, by intimal thickening occurring after vascular recanalization procedures. "Vascular recanalization" refers to a procedure for restructuring a vessel, including, for example, ablative and angioplastic procedures. "Inhibiting restenosis" refers to substantially inhibiting smooth muscle cell proliferation in a statistically significant fashion; for example, inhibiting smooth muscle cell proliferation by about 50%, or preferably by about 80 %, and more preferably by about 95% as compared to an untreated control. It is not essential, however, that the methods of the present invention result in the inhibition of all proliferating smooth muscle cells. It is sufficient that smooth muscle cell proliferation is arrested at the site of treatment such that the remaining fraction of proliferating smooth muscle cells is insufficient to cause restenosis. "Inhibiting restenosis" also refers to reduction in intimal thickening and reduction in the narrowing of luminal diameter.

Modes of Administration

The methods of the present invention include administering compounds for use in the invention by local delivery systems. The compounds for use in the present invention may be administered by local delivery at a time proximal to the recanalization procedure or at a time after the recanalization procedure. The

The efficacy of drug delivery by an infusion sleeve on the arterial architecture of a vessel is a function of proximal delivery pressure. In one embodiment, the effect of proximal pressure on delivery of compounds used in the methods of the present invention by an infusion sleeve catheter can be determined in vitro by histological evaluation of the treated artery by known methods. In one non-limiting example, a proximal pressure of between about 50 to 200 psi may be used to deliver, by an infusion sleeve catheter, the compounds for use in the methods of the present invention, preferably, between about 100 to about 150 psi, and most preferably, between about 50 to 100 psi.

In another embodiment, the compounds of the invention may be administered locally by diffusion-based catheter systems, including for example, double balloon, dispatch, hydrogel and coated stent catheters. The methods of the invention also include local administration of the compounds used in the methods of the present invention by mechanical device-based catheter systems, including for example, iontophoretic balloon catheters.

The ability to locally deliver the compounds used in the present invention may be evaluated in vivo using known animal models, including for example, acute canine coronary models. For example, a compound for use in the methods of the present invention is administered by local delivery to a canine at a site of injury. The canine is sacrificed and then examined by known methods, including, for example, fluorescence microscopy.

Optimum conditions for delivery of the compounds for use in the methods of the invention may vary with the different local delivery systems used, as well as the properties and concentrations of the compounds used. Conditions may be optimized for inhibition of smooth muscle cell proliferation at the site of injury such that significant arterial blockage due to restenosis does not occur, as measured, for example, by the proliferative ability of the smooth muscle cells, or by changes in the vascular resistance or lumen diameter. Conditions which may be optimized include, for example, the concentrations of the compounds, the delivery volume, the delivery rate, the depth of penetration of the vessel wall, the

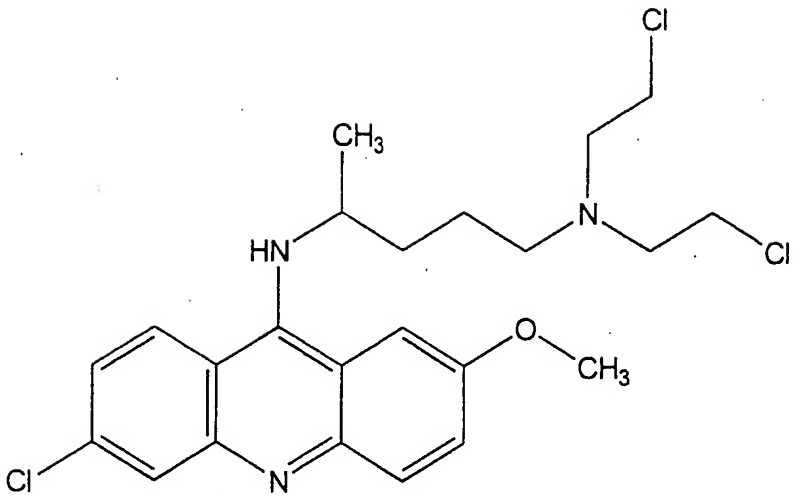
Vaccine Research 5:29-39 (1996); and PCT WO 97/07674, the disclosures of which are incorporated herein by reference.

The methods of the invention also include compounds containing functional groups that are the "equivalent of mustards." Mustard group equivalents are defined as those in which the halide of the mustard is replaced with a different leaving group, such as mono or bis mesylethylamine groups, mono mesylethylsulfide groups, mono or bis tosylethylamine groups, and mono tosylethylsulfide groups; and/or those which react by a mechanism similar to that by which mustards react, such as an epoxide.

The methods of the present invention also comprise the administration by local delivery to the site of injury of compounds that include an effector group covalently bonded to a nucleic acid binding ligand.

A "nucleic acid binding ligand", (or "anchor") is herein defined as a group which has an affinity for and can bind to nucleic acids non-covalently. While not limited to any particular mechanism, it is believed that the nucleic acid binding ligand functions as a carrier (or anchor) that targets (or directs) the molecule to nucleic acids, interacting non-covalently therewith. The anchor-effector arrangement enables the compounds to bind specifically to nucleic acid (due to the anchor's binding ability). This brings the effector into proximity for reaction with the nucleic acid. There are several modes of binding to nucleic acids. Compounds which bind by any of the following modes, combinations of them, or other modes are considered nucleic acid binding ligands. While the invention is not limited to the following compounds for use in the methods of the present invention, some examples of nucleic acid binding ligands are:

a) intercalators, such as, acridines (and acridine derivatives, e.g. proflavine, acriflavine, diacridines, acridones, benzacridines, quinacrines), actinomycins, anthracyclinones, rhodomycins, daunomycin, thioxanthenones (and thioxanthenone derivatives, e.g. miracil D), anthramycin, mitomycins, echinomycin (quinomycin A), triostins, ellipticine (and dimers, trimers and analogs thereof), norphilin A, fluorenes (and derivatives, e.g. flourenones,



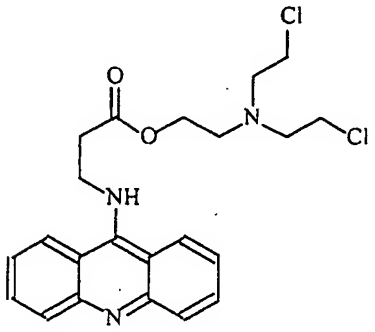
Other examples of compounds for use in the methods of the present invention which include an effector group and an anchor are disclosed in PCT WO 96/14737, PCT WO 96/39818, and U.S. Patent No. 5,559,250, the disclosures of which are incorporated by reference herein.

The methods of the present invention further comprise the administration by local delivery to the site of injury of compounds that include an anchor group covalently bonded to a frangible linker, which is covalently bonded to an effector group, referred to herein as a FRALE.

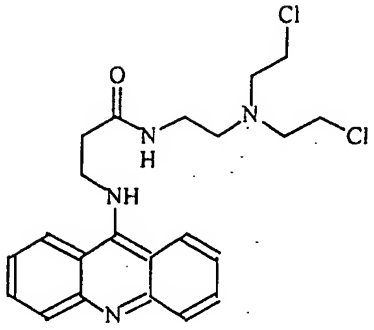
The term "frangible linker" refers to a moiety which serves to covalently link the anchor and effector, and which will degrade under certain conditions so that the anchor and effector are no longer linked covalently. Like the alkylating compounds described above, the anchor-frangible linker-effector arrangement enables the compounds to bind specifically to nucleic acid. This brings the effector into proximity for reaction with the nucleic acids. Compounds including an anchor-frangible linker-effector arrangement are disclosed in PCT publication WO 98/30545, the disclosure of which is incorporated herein by reference.

Examples of frangible linkers for use in the methods of the present invention, include, but are not limited to, moieties that include functional groups such as ester (where the carbonyl carbon of the ester is between the anchor and

(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl amide includes a frangible linker with an amide functionality.



β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester



β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl amide

The reactivity of the compounds for use in the methods of the present invention may be enhanced by making modification on the effector moiety, or in the case of the FRALE compounds, on either the effector or linker moieties. Modifications on the effector moiety may change the compounds reactivity towards alkylation, while modifications on the substituents of the linker group may change the reactivity toward hydrolysis of this group. An enhancement of the alkylation reaction results in a faster formation of DNA adducts which is thought to correlate with cellular inactivation. An enhancement of the hydrolysis reaction is thought to result in a faster decomposition of the compound to less toxic and less mutagenic end-products. Conditions which may be optimized for enhancing the reactivity of the compounds for use in the methods of the present invention include the equilibration rate of the compound intra- and extracellularly, the rate of reaction with the nucleic acid of the cells and the rate of the ester hydrolysis. In one embodiment, the compounds for use in the present invention have a fast and favorable equilibration towards the intracellular space, complemented by a reactive effector group with a comparable reaction rate. In another embodiment, the hydrolysis rate of the linker, although fast, is slightly slower than the alkylation rate of the compound. In one non-limiting example, the

The alkylating compounds can be provided with or without adjuvants. In addition, subsequent to the administration of the alkylating compounds, a gel may be delivered to the treated site. The gel acts to coat the compounds so as to prevent the compounds from being washed away by the naturally occurring biological fluids for about 3 hours from the time of administration. Further, the alkylating compounds can be introduced alone, or in a "cocktail" or mixture of several different alkylating compounds.

In a non-limiting example, the compounds of the present invention can be administered at a concentration of between about 10 nM to about 100 μM, more preferably between about 100 nM to about 1 μM, and most preferably less than about 300 nM.

Evaluating the Effectiveness of Methods of the Present Invention in Inhibiting Smooth Muscle Cell Proliferation in a Blood Vessel Subjected to Recanalization

The effectiveness of the methods of the present invention can be measured by methods that are known in the art, including for example, methods for measuring the inhibition of smooth muscle cell proliferation (*e.g.*, measuring the uptake of [^{3}H]-thymidine by smooth muscle cells) or methods for measuring changes in arterial blockage.

For example, the effectiveness of the methods of the present invention can be measured by the uptake of [^{3}H]-thymidine by smooth muscle cells *in vitro* following treatment according to the methods of the present invention. The conditions used in the methods of the present invention are such that the uptake of [^{3}H]-thymidine by smooth muscle cells is reduced by about 50% as compared to an untreated control, preferably by about 80%, most preferably by about 95%.

In another example, the effectiveness of the methods of the present invention is measured *in vitro* by assaying for production of human cytokines that are connected with cell proliferation and response to injury, including for example, the production of interleukin 1β (IL-1β), interleukin 8, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and

assessing the effectiveness) the animal is examined *e.g.* for compound distribution, vessel lumen diameter, or intimal thickness by known methods, including, for example, fluorescent microscopy and ultrasound.

In one example, the effectiveness of the methods of the present invention is measured *in vivo* in an animal model by measurment of luminal diameter of a vessel. For example, luminal diameter is measured upon follow-up angiogram six months after initial treatment. The diameter of the lumen is measured, for example, by angiography, intravascular ultrasound imaging, or any other method known to those of skill in the art. Luminal diameter upon follow-up angiogram, for example, six months after initial treatment, decreases by no more than about 50%, more preferably by no more than about 10%, and most preferably by no more than about 5%, relative to the diameter immediately after treatment.

In another example, the effectiveness of the methods of the present invention are measured *in vivo* in animal models by the thickening of the intima, for example, upon follow-up six months after initial treatment. The thickness of the intima is measured by methods known to those of skill in the art, such as sectioning and histological analysis of treated vessels. The intimal thickness upon follow-up, for example, six months after initial treatment, increases no more than about 50%, more preferably no more than about 10%, and most preferably no more than about 5% relative to the thickness immediately after treatment.

In another example, the effectiveness of the methods of the present invention are evaluated *in vitro* by observation of human smooth muscle cell proliferation as a function of treatment dose and time of treatment. The smooth muscle cells can be cultured following treatment and observed under a microscope in order to asign a score which indicates whether the cells are proliferating and whether they are still viable cells. Based on the scoring scale of Example 1, a compound for use in the method of treatment is preferred in which the treated cells score +/-, 1+, or 2+, more preferably 1+. In a preferred embodiment, the compounds of the present invention at concentrations between about 100 nM and 10 μM exhibit these scores, more preferably between about

with an electrophilic group on the alkylating compound. Exemplary nucleophilic groups include thiol, thioacid, dithioic acid, thiocarbamate, dithiocarbamate, amine, phosphate, and thiophosphate groups. The quencher may be, or contain, a nitrogen heterocycle such as pyridine. The quencher can be a phosphate containing compound such as glucose-6-phosphate. The quencher also can be a thiol containing compound, including, but not limited to, glutathione, cysteine, N-acetylcysteine, mercaptoethanol, dimercaprol, mercaptan, mercaptoethanesulfonic acid and salts thereof, *e.g.*, MESNA, homocysteine, aminoethane thiol, dimethylaminoethane thiol, dithiothreitol, and other thiol containing compounds. One preferred quencher is glutathione. Other thiol containing compounds include methyl thioglycolate, thiolactic acid, thiophenol, 2-mercaptopyridine, 3-mercapto-2-butanol, 2-mercaptobenzothiazole, thiosalicylic acid and thioctic acid. Exemplary aromatic thiol compounds include 2-mercaptobenzimidazolesulfonic acid, 2-mercapto-nicotinic acid, napthalenethiol, quinoline thiol, 4-nitro-thiophenol, and thiophenol. The quencher also can be a peptide compound containing a nucleophilic group. For example, the quencher may be a cysteine containing compound, for example, a dipeptide, such as GlyCys, or a tripeptide, such as glutathione.

The quenchers can be administered to the individual by systemic or local delivery. If the quencher is administered to the individual systemically, the quencher can be added, for example, prior to, in proximal time with, or after the administration of the compounds for use in the methods of the invention. If the quencher is administered by local delivery, the quencher may be added, for example, in proximal time with, or after the administration of the compounds for use in the methods of the invention. In either situation it is important that the compounds for use in the methods of the invention have had sufficient exposure time with the site of injury to cause the desired inhibition of smooth muscle cell proliferation before the quenching effect takes place.

For use in the methods of the present invention quenchers are preferred which substantially decrease the concentration of reactive electrophilic alkylating

Three-fold serial dilutions of a stock solution of β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester were made in unbuffered saline solution (BBS, blood bank saline) to generate concentrations from 3 mM to 23 pM. Dilutions were made immediately prior to use to avoid the possibility of compound degradation.

The growth medium was removed from the Vero cell-containing plates. The cell monolayer was then washed several times with between 2 and 5 mL of BBS to remove all medium constituents which may quench the activity of β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. The BBS was removed from all wells and 100 µL of each dilution of β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester, prepared as described above, was added to 1 well each of the 6-well plates. The plates were gently rocked back and forth for the 3 minute exposure period to ensure uniform distribution of the β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester and to guarantee that the monolayer did not dry due to exposure to air.

At the conclusion of the three minute incubation, between 4 and 6 mL of Phosphate Buffered Saline at pH 7.2 (PBS; Life Technologies) was added to each well and immediately removed by aspiration, followed by subsequent washing with PBS to ensure complete removal of the β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. This was followed by the addition of growth medium which contained 0.75% molten Seaplaque® Agarose (FMC, Rockland ME) to ensure that killed cells remained in close association with the monolayer and could be scored. Without the addition of the agarose overlay, dead cells detach from the plastic substrate, float off and are unavailable for evaluation. The medium also contained 10% FBS for growth promotion, to permit proliferation of cells insufficiently affected by the treatment. The plates were then briefly allowed to cool at approximately 22°C to allow the agarose to harden. Treated cells were then incubated in a 37°C humidified 5% CO_2 incubator (Forma Scientific, Marietta OH).

Results:

Growth Scoring of β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester treated cultures:

β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester concentration	Vero Cells	T/G/ HA-VSMC
3 mM	-	na
1 mM	-	-
333 μM	-	-
111 μM	-	-
37 μM	-/+	-
12 μM	+/-	-
4.1 μM	1+	-/+
1.3 μM	1+	1+
457 nM	1+	1+
152 nM	2+	2+
50 nM	3+	2+
17 nM	3+	3+
5.6 nM	3+	3+
1.9 nM	3+	3+
600 pM	4+	4+
200 pM	4+	4+
70 pM	4+	4+
23 pM	4+	na

Score	Effects of treatment
-	Lethal treatment, no cells left viable, all rounded, shriveled and dead.
-/+	Some treated cells remain viable, about equal amount of clearly dead cells.
+/-	Most cells remain viable, detectable number of dead cells
1+	All cells appear viable, same density as when treated.
2+	Minimal proliferation, compared to control.
3+	Moderate proliferation compared to control.
4+	Maximal growth, unaffected by treatment.

The results demonstrate that β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester inhibited the increase in the number of cultured Human Aortic Smooth Muscle Cells and Vero cells in a dose-dependent manner.

lines), TES and HEPES buffers, ECGS (endothelial cell growth supplement), freshly prepared ascorbate, glutamine, penicillin-streptomycin, and 10% FBS (Gemini) (all other reagents, Life Technologies, or Cellgro). Cells were cultured in 96-well plates.

Ten-fold serial dilutions of β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester and QM were made in unbuffered saline solution (BBS, blood bank saline) to generate concentrations from 100 μM to 1 nM. Similarly, serial dilutions of chlorambucil, busulfan, melphalan, and mechlorethamine were prepared to generate concentrations of from 1 mM to 10 nM. The stocks were dissolved and diluted immediately prior to use to avoid the possibility of compound degradation.

The growth medium was removed from the smooth muscle cell (SMC)-containing plates. The cell monolayer was then washed with BBS to remove all medium constituents which may quench activity of the compound. The BBS was removed from all wells and 100 μL of each dilution of compound was added to one well each of the tissue culture plates.

Incubation of SMC in the presence of compound was conducted for 15, 30, 60, 90 and 180 seconds. At the conclusion of the incubation, Phosphate Buffered Saline at pH 7.2 (PBS; Life Technologies) was added to each well and immediately removed by aspiration, followed by subsequent washing with PBS to ensure complete removal of each compound solution. This was followed by the addition of growth medium containing 10% FBS for growth promotion, to allow proliferation of cells insufficiently affected by the treatment. Treated cells were then incubated in a 37°C humidified 5% CO_2 incubator (Forma Scientific, Marietta OH).

Growth of cell monolayers was scored eight days following treatment, when a differential growth rate could be determined, using an inverted microscope. Growth was scored subjectively by the careful evaluation of each treated culture and was aided by trypan blue dye exclusion, as follows. Growth medium was removed by aspiration, then 150 μL of a 1:4 dilution of trypan blue

Chlorambucil

concentration	Time: seconds 0	15	30	60	90	180
1 mM	4+	-	-	-	-	-
100 µM	4+	+/-	+/-	+/-	+/-	-/+
10 µM	4+	1+/2+	1+/2+	1+	1+	+/-
1 µM	4+	4+	4+	4+	4+	3+
100 nM	4+	4+	4+	4+	4+	4+
10 nM	4+	4+	4+	4+	4+	4+
0	4+	4+	4+	4+	4+	4+

Busulfan

concentration	Time: seconds 0	15	30	60	90	180
1 mM	4+	4+	4+	4+	4+	4+
100 µM	4+	4+	4+	4+	4+	4+
10 µM	4+	4+	4+	4+	4+	4+
1 µM	4+	4+	4+	4+	4+	4+
100 nM	4+	4+	4+	4+	4+	4+
10 nM	4+	4+	4+	4+	4+	4+
0	4+	4+	4+	4+	4+	4+

Melphalan

concentration	Time: seconds 0	15	30	60	90	180
1 mM	4+	2+	2+	2+	2+	1+
100 µM	4+	3+	3+	3+	3+	3+
10 µM	4+	4+	4+	4+	4+	4+
1 µM	4+	4+	4+	4+	4+	4+
100 nM	4+	4+	4+	4+	4+	4+
10 nM	4+	4+	4+	4+	4+	4+
0	4+	4+	4+	4+	4+	4+

the medium is removed and 2 ml of phosphate buffered saline is added to wash growth medium from the monolayer. The PBS is then removed and replaced by 1.5 ml/well of an identically formulated F12K medium that lacks FBS, to induce the cells to enter the resting G_o state. This treatment synchronizes the cells so that this test will accurately assess the ability of β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester to arrest growth cycle progression.

After 48-72 hours of synchronization, the SMCs are treated with β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester over a concentration range of 300 nM to 1 μM. A minimum of 30 minutes is allowed for the equilibration of drug. A negative control for 10% fetal bovine serum and an untreated fetal bovine serum stimulated control is included.

Following incubation, the medium is removed and replaced by medium containing 10% fetal bovine serum to allow the proliferation of cells unaffected by the treatment. Cultures are then incubated at 37°C with growth medium changes every 2-3 days to sustain cell growth.

Analysis of Cell Count following Treatment of Human Aortic Smooth Muscle Cells.

Cell count for each sample, taken by direct counting with a hemacytometer, is measured at several time points over a 28 day period. At every time point and media replacement, a 1 ml aliquot of each sample is removed and preserved at -80° C for cytokine studies described below. The remaining recovered cells are preserved at -80° C for subsequent analysis of nuclear DNA content by flow cytometry.

Analysis of Cell Cycle following Treatment of Human Aortic Smooth Muscle Cells.

Measurements of SMCs cell cycle are performed by flow cytometric analysis of nuclear DNA content in cryopreserved samples according to an established protocol for the Becton-Dickinson FACScan, as further detailed in the literature with specific reference to SMCs. See, *e.g.* March et al., *Circulation* 87, 184-191 (1993).

CLAIMS

What is claimed is:

1. A method for inhibiting cell proliferation at a site of vascular injury, the method comprising local administration of an alkylating compound to said site of injury.

2. The method according to claim 1, wherein the alkylating compound comprises a chemical group which forms a covalent bond with a nucleic acid.

3. The method according to claim 2, wherein the chemical group is selected from the group consisting of mustards, mustard intermediates and mustard equivalents.

4. The method according to claim 3, wherein the mustard is an aliphatic mustard.

5. The method according to claim 2, wherein said chemical group is covalently bonded to a nucleic acid binding ligand.

6. The method according to claim 5, wherein the nucleic acid binding ligand is selected from the group consisting of intercalators, minor groove binders, major groove binders, electrostatic binders, nucleic acids, and derivatives thereof.

7. The method according to claim 6, wherein the intercalator is an acridine or acridine derivative.

8. The method according to claim 7, wherein the compound is a quinacrine mustard.

9. The method according to claim 2, wherein said chemical group is covalently bonded to a frangible linker, wherein the frangible linker is covalently bonded to a nucleic acid binding ligand.

10. The method according to claim 9, wherein said frangible linker is selected from the group consisting of esters, thioesters, thionoesters, dithioic acids, sulfates, sulfonates, phosphates, phosphonates and amides.

11. The method according to claim 9, wherein the alkylating compound is β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester.

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(54) Title: USE OF ALKYLATING COMPOUNDS FOR INHIBITING PROLIFERATION OF ARTERIAL SMOOTH MUSCLE CELLS

(54) Titre: INHIBITION DE LA PROLIFERATION DES CELLULES MUSCULAIRES LISSES ARTERIELLESH MUSCLE CELLS

(57) Abstract

The present invention provides methods and compositions for inhibiting the proliferation of smooth muscle cells at a site of vascular injury. The methods include intravascular administration of a reactive compound to the site of injury, without the requirement for activation or sustained release of the compound.

(57) Abrégé

L'invention concerne des procédés et des compositions qui permettent d'inhiber la prolifération des cellules musculaires lisses sur un site de blessure vasculaire. Les procédés décrits consistent notamment en une administration intravasculaire de composé réactif sur le site de la blessure, sans qu'il soit nécessaire d'activer ou de prolonger la libération du composé.

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INTERNATIONAL SEARCH REPORT

Inter ional Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	M.G. SIROIS: "Rat arterial wall retains myointimal hyperplastic potential long after arterial injury" CIRCULATION, vol. 96, no. 4, 1997, pages 1291-1298, XP000874225 the whole document	1-22

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Continuation of Box I.2

The expression "alkylating compound" encoampases an extremely large number of possible compounds.. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds mentiuoned in the pharmacological examples.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.